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ANTIBACTERIAL ACTIVITY AND IN VITRO CALLUS INDUCTION OF HYBANTHUS ENNEASPERMUS (L.) F. MUELL

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ABSTRACT

The purpose the research was carried out to evaluate the antibacterial property and in vitro response from three explants (shoot tips, nods segment and leaves) of Hybanthus ennaspermus. The range of MIC and Zone of inhibition for V. cholera in Diethyl ether extract is (6.25µg/ml), P. aeruginosa in Ethanol extract (3.13 µg/ml), K. pneumonia in Diethyl ether extract (3.12 µg/ml), B. subtilis in Diethyl ether extract (3.13 µg/ml), S. aureus in Ethanol extract (3.13 µg/ml) and E. coli in Ethanol extract (0.78 µg/ml). Among the six pathogens tested, V. cholera was least vulnerable to the extracts followed by S. aureus, P. aeruginosa, B. subtilis, E. coli. and K. pneumonia. Among the different solvents tested, diethyl ether was more active and the rest of the extract showed moderate inhibitory effect to the test pathogens. Maximum callus Induction was noted in MS medium fortified with BA (1.0mg/L) + NAA (0.4mg/L). Induction and formation of callus was also succeeded by adding 2,4-D (1.5mg/L) and NAA (0.6mg/L). Beyond the optimum doses of 2,4-D leads to toxic and reduced the callus formation rate. It is observed that the leaves, nodes, and shoot tip explants are best source for callus induction and produced the callus after 4 weeks of culture. This study found that callus induction rates are strongly reliant on the explant type rather than the media employed. The nodal explants also induced the callus but the percentage callus initiation was higher in leaf explants. It was observed that very low response occurred in MS medium encompassing BA and 2, 4-D along with NAA, by using shoot tips as an explant.

KEYWORDS: Antibacterial Activity, Callus Induction, In Vitro Culture & MIC

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Original Article

INTRODUCTION

Owing to the wide range of climatic conditions found in various ecological regions, India is one of the most important biodiversity places on the planet. This biodiversity provides a supply of life-saving medicine and high-value new molecules. The flora of India is abundant in herbal medicines. In India, about 7000 distinctive species of plants from diverse habitats are reported to be used for therapeutic purposes. The Charaka Samhita (1000 B.C.) is one of the first discourses of Indian medicine, and it discusses the use of nearly 2000 plants for therapeutic purposes. Most of the medicines are procured from plants amongst 90% are collected from Indian wilds. Ayurveda, Charaka Samhita, and Susruta Samhita were early Hindu treatises that addressed with plants quite often in connection to treatments (Rajendra and D'souza, 1997).

According to the World Health Organization (WHO), natural drugs are used by 80% of the world's population for primary health care, particularly in developing countries, due to their safety, performance, and public acceptance (Kamboj, 2000). India offers a wealth of traditional medicinal medical knowledge that has been chronicled and used for generations. (Sharma, 1999).

In 1989, the World Health Assembly addressed the discussion and endorsed a statement (WHA) stating that herbal therapy is vital to individual and community health. Plant-based substances have long been used as a source of therapeutic ingredients. People have been using the significant biochemical properties of diverse herbs for the various ailments since the birth of civilisation. Plants have always been the most predominant supplier of medications for the bulk of the world's population, with plant products accounting for roughly 25% of all prescription medicines. So many medicinal plants which were ignored in the past years, have been over exploited in recent years. As a result, the consumption of plant hunters grows, while the number of plants still found in the wild diminishes. As a result, many plants have been declared to be endangered.

Antibiotic resistance continues to emerge in both dangerous and agile microorganisms, pushing scientists to develop new drugs and treatment targets on a regular basis. The pharmacist has only launched a few novel antimicrobial drugs in the last few years, none of which have improved the action against multidrug-resistant bacteria (Conlon et al., 2004). Numerous studies have endeavored to identify innovative, potent antimicrobial compounds free of resistance and rate in reaction to the rise and spread of microscopic creatures resistant to diverse antibiotics, in addition to the increasing focus on health-care costs. Plants have been found to contain a variety of phytochemicals, many of which have antibacterial effects (Cowan, 1999). Plant-based treatments have been utilised in conventional medicine across the globe for many years, and there is strong demand in plants as antibacterial agents (Chariandy et al., 1999).

Naturally and commercially plant propagates by the means of seeds but propagation through seed poses difficulties because of poor germination and deprived viability, and it also lack the innate ability to replicate vegetatively (Sen and Sharma, 1991) but the seed sustainability is restricted to a year (Rani and Grover, 1999), rendering long-term seed storage futile (Farooqi and Sreeramu, 2004). The over-exploitation of this plant due to their multidimensional medicinal properties and a constituent in various formulations in different system of medicine, the plant is in serious danger of disappearing (Abhyankar and Chinchanikar, 1996). Though since collected seed has a low survivability, an alternative method of regeneration is required for steady flow at the economic level. There seems to be a solid necessity strategic knowledge of how to repopulate, conserve, and utilize this species using modern biotechnological methods via plant tissue culture approaches. One method for tissue culture approaches for rapid clonal proliferation is the micropropagation system and it has developed a key aspect of marketable propagation. (Dirr and Heuser, 1987).

The objective of commercial propagation is to reproduce copies of an original parent plant in a very high multiplication rate. Many commercial laboratory nurseries have the capacity to generate millions and millions of micro propagated plants in a short time. Tissue culture systems have been used to strengthen the genetics of economically powerful plants (Scowcraft, 1977; Metha and Mohan Ram, 2007 Plant development using comprehensive in vitro technologies necessitates the regeneration of whole plants from tiny portions of tissue, single cells, or protoplasts (Jacobsen and Kysely, 1984).

H. enneaspermus member of the *Violaceae* family of and it’s a rare perennial herb distributed in warmer region in India. Local tribes, peasants, and herbalists call this ethnobotanical herb 'Ratanpurus,' and it is recognised to have special pharmacological characteristics. Herbal medicine makes use of the libido, antiulcer, and restorative properties of formulations made from the plant's leaves and sensitive stalks. The root is diuretic and is used as an infusion to treat syphilis and bladder infections. The tribal groups utilise the fruits and leaves as antitoxins for scorpion and cobra attacks.

Based on the above information and the importance of the study plant in the field medicine in all the systems, the current experiment has been carried out with the underlying goals are to study the antibacterial activity of different solvent extracts using 96 well plate micro dilution bio-assay method and to study the *in-vitro* response of *Hybanthus enneaspermus* L. Muell.

MATERIALS AND METHODS

One of the important medicinal plant *Hybanthus enneaspermus* belonging the family Violaceae was selected in the present study.

Plant Collection and Preparation of Samples

The fresh plants were collected from A.V.C. College Campus and immediately transported to the laboratory. The plants were completely cleaned with running tap water to eliminate the dirt and then shade dried at room temperature for 3–5 days before being oven for 2 hours at 40° C. The well dried samples were made in to powder with help of grinder and those powder samples were packed and stored at 4° C for further use.

Test Organisms

Six bacterial cultures viz, *Vibriae cholera, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtili and Escherichia coli* were used for this study and the culture were procured from Microbial Type Culture Collection centre (MTCCC), Chandigarh.

Preparation of Extracts

A sequence of extraction method was followed. The powdered samples (100 g) were extracted for 24h in 500 ml of Ethanol, Acetone and Diethyl ether separately. The extracts were filtered with assistance of Whatmann No. 1 filter paper. Using a rotary flash evaporator, all extracts were concentrated.

Minimum Inhibitory Concentration (MIC) Method

The microdilution bioassay was used to identify the MIC of compounds for antibacterial activity (Eloff 1998). Overnight cultures were inoculated with around 106 CFU/ml in sterile Mueller–Hinton (MH) broth and incubated at 37°C with an orbital shaker. Individual extracts were resuspended in their respective solvents at specified quantities (50 mg/ml). In a 96-well microlitre plate, 100 µl of individual extract were serially diluted two-fold with sterile distilled water for each of the five bacterial strains. A two-fold serial dilution of Streptomycin (Sigma Aldrich) (0.1 mg/ml) served as a positive control against each microorganism. Each bacterial culture was given 100 µl to each well. In this study, water and various solvents were used as negative controls. The plates were and incubated at 37 ◦C for 24 h and the growth of bacterium was measured using 50 µl of 0.2 mg/ml p-iodonitrotetrazoliu chloride (INT) (Sigma–Aldrich) incubated at 37°C for 2 hours. The MIC values were determined based on the dosage level in the last well where colour change was not noticed after addition of INT indicator, because the colour free tetrazolium salt is biologically transformed to a red product in the existence of live organisms. A reddish-pink colour suggested bacterial development in the wells. The test was reiterated thrice with two replicates each time.

Source of Explants for *In-Vitro* Response

The plant was collected from A.V.C College Campus, Mannampandal, Mayiladuthurai. The plant was collected and

washed with tap water to eliminate the soil and dirt particles. The leaf, node and shoot tip were cut from the plant materials and used as source of explant.

Explants Sterilization

The explants were completely washed under running tap water for 20 minutes before being submerged in a detergent solution (Teepol-5 percent v/v) for 10 minutes. The explants were then properly cleansed with double distilled water after being cleaned in running tap water till all traces of soap solutions were gone. Further the explants were disinfected with 0.1 % v/v $HgCl_2$ solution for 5 mins and sub sequentially washed thrice with sterile water (Sathish et al, 2019; Sathish et al, 2020).

Culture Medium

In the current research, MS (Murashige and Skoog, 1962) medium with Vitamin B5 (Gamborg et al., 1968) augmented with diverse concentration and amalgamations of diverse plant growth hormones were employed. On nutritional media with a high sucrose concentration, many bacteria can grow. These bacteria multiplied far faster in the media than the cultured explants, eventually destroying it. In order to keep a the culture tubes sterile the medium was autoclaved. As a result, culture tubes are left open until the medium's agar solidifies. After that, the tubes were moved to an inoculation chamber where they were inoculated.

Callus Induction

Callus induction was initiated by culturing the leaf, node and shoot tip explants. Various concentrations of growth hormones were utilized to test the optimal supplement for callus initiation on MS medium with BA (0.5 -2.5mg/L) alone, 2,4-D (0.5 -2.5mg/L) alone, and amalgamations of 2,4-D (0.5 -2.5mg/L) + NAA(0.2- 1.0mg mg/L), BA (0.5 -2.5mg/L)+NAA(0.2-1.0mg mg/L). The combinations of the diverse plant hormones of callus induction from different explants were given in the Table 2 to Table 7.

All treatment consisted of twenty culture tubes containing single explants, which were repeated three times. At 25 2° C, the cultures were conserved under a photoperiod of 16 hours of light per day (2400 Lux). After four weeks in culture, multiple shoot was measured by calculating the proliferating shoots that were 2.0 cm or longer.

RESULTS

Antibacterial Activity

In the current research, the preliminary antibacterial effect of *Hybanthus enneaspermus L. Muell* was carried out using the minimum inhibitory concentration method. The antibacterial activities of different extracts of *Hybanthus enneaspermus L. Muell* on six human pathogens are summarized in table 1. The results indicated all the extracts possess antibacterial activity (Plate – II & III). MIC was calculated for all the drugs over the test pathogens. The range of MIC to inhibit *V. cholera* (6.25µg/ml) in Diethyl ether extract, *P. aeruginosa* (3.13 µg/ml) in Ethanol extract, *K. pneumonia* (3.12 µg/ml) in Diethyl ether extract, *B. subtilis* (3.13 µg/ml) in Diethyl ether extract, *S. aureus* (3.13 µg/ml) in Ethanol extract and *E. coli* (0.78 µg/ml) in Ethanol extract were observed. The results showed all the extracts possess antibacterial activity. For each of the test pathogens, the MIC was calculated.

Leaf Explant Culture

Generally induction of callus happened either at the cut ends (or) whole of the leaf segments callused simultaneously and

within three weeks the entire explant transformed into a either a fragile or compact calli. Leaf The maximum callus induction (90%) noted on leaf explants culture holding MS medium amended with 2,4-D (1.0mg/L)+NAA(0.4mg/L).

Induction of callus was also recorded on MS medium assisted with BA (1.0mg/L) and NAA(0.4mg) BA (1.0mg/L) alone augmented medium showed less response (70%). The response of the various growth hormones for stimulation of callus from leaf explant explained in the Table 2 &Table 3.

Nodal Explant Culture

Callus induction was persuaded from nodal explant on MS medium containing diverse concentration of 2,4-D (0.5-2.5 mg /L) alone or in amalgamation with NAA (0.2-1.0 mg/L) however, nodal explants inoculated on MS medium amended with 2,4-D(0.1mg/L) + NAA (0.4mg/L) showed the best results (80%). Callus response was about 70% by using 2,4-D alone in the culture medium.

Callus formation (70%) was also recorded on MS medium augmented with BA (1.0mg/L) and NAA (0.4mg/L) in MS medium encompassing BA (1.0mg/L) give less response (60%) by using nodal explant. The response of various plant hormones on callus formation from nodal explant is given in the Table 4 & Table 5.

Shoot tip Explant Culture

Callus of the stem segment occurred on MS medium augmented with 2,4-D (0.5-2.5mg/L) alone or in amalgamation with NAA(0.5-2.5mg/L).Best growth of callus however occurred on MS + 2,4-D (1.5mg/L) + NAA(0.6mg/L) at cut ends after 2-3 weeks of culture.MS medium comprising 2,4-D alone also showed callus initiation in 2,4-D (1.5mg/L).

The stem segments also callused on MS medium encompassed with BA (1.0 mg/L) + (0.4 mg/L), maximum response occurred (70%), when stem explants were inoculated on MS + BA (1.0 mg/L) but growth of callus was less compared to MS + BA + NAA. Impact of diverse growth hormones on callus formation from shoot tip explant is summarized in Table 6 and Table 7.

DISCUSSIONS

The results showed all the extracts possess antibacterial activity. MIC was assessed for all the compounds over the test pathogens. Parallel outcomes were recorded by Nadia Alam et al., (2012). The range of MIC to inhibit *V. cholera* (6.25µg/ml) in Diethyl ether extract, *P. aeruginosa* (3.13 µg/ml) in Ethanol extract, K. *pneumonia* (3.12 µg/ml) in Diethyl ether extract, *B. subtilis* (3.13 µg/ml) in Diethyl ether extract, S. *aureus* (3.13 µg/ml) in Ethanol extract and *E. coli* (0.78 µg/ml) in Ethanol extract were observed. Raveendra Retnam and John De Britto (2007) studied the antibacterial effect of *Hybanthus enneaspermus* and obtain the efficiency of different solvents and benzene extract was more active. In this present investigation also aqueous methanol is more active followed by methanol, dichloromethane + methanol and dichloromethane were more active and the rest of the extract showed moderate inhibitory effect to the test pathogens.

In the current experiment highest callus induction obtained from leaves in MS medium fortified with BA (1.5mg/L) and NAA (0.4mg/L).The three explants (shoot tips, nodes and leaves) produced high to moderate percentage of callus. In contrast, high percentage of callus initiation was achieved from leaves on MS basal medium augmented with combination of BA and NAA. The concentration of BA, presence or lack of NAA and their interactions influenced induction frequency and the growth of leaf derived callus. Similar reports are available for the seed derived callus stimulation on MS medium added with NAA (2.6µm) and BA (2.2µm) (Patnaik *et al.*,2016).

Table 1: Antibacterial Activity of Ethanol, Acetone and Diethyl ether Extract of Hybanthus Enneaspermus

Sl. No.	Microorganism	Minimum Inhibition Concentration (µg/ml)		
		Ethanol Extract	Acetone Extract	Diethyl ether Extract
01	*V. cholera*	1.65	3.13	6.25
02	*P. aeruginosa*	3.13	0.39	0.78
03	*K. pneumonia*	1.56	0.19	3.12
04	*B. subtilis*	1.56	1.56	3.13
05	*S. aureus*	3.13	0.78	0.19
06	*E. coli*	0.78	0.19	0.09

Table 2: Influence of Diverse Level of BA and NAA on Callus Formation from Leaf Explant of Hybanthus Enneaspermus

Growth Regulators (mg/L)		Culture Response in Percentage	Callus Response
BA	NAA		
0.5	-	50	++
1.0	-	70	+++
1.5	-	60	++
2.0	-	50	++
2.5	-	50	++
0.5	0.2	60	++
1.0	0.4	80	+++
1.5	0.6	70	+++
2.0	0.8	60	++
2.5	1.0	30	+

0 – 20% - No Response (or) Very Less response, 21-40% + Poor Response, 41-70% ++ Moderate Response, 71-100% +++ Good Response

Table 3: Influence of Diverse Concentration of 2,4-D and NAA on callus Formation from Leaf Explant of Hybanthus Enneaspermus

Growth Regulators (mg/L)		Culture Response in Percentage	Callus Response
2,4-D	NAA		
0.5	-	30	+
1.0	-	80	+++
1.5	-	70	+++
2.0	-	50	++
2.5	-	30	+
0.5	0.2	70	+++
1.0	0.4	90	+++
1.5	0.6	60	++
2.0	0.8	50	++
2.5	1.0	20	-

0 – 20% - No Response (or) Very Less response, 21-40% + Poor Response, 41-70% ++ Moderate Response, 71-100% +++ Good Response

Table 4: Influence of Diverse Level of BA and NAA on Callus Induction from Nodal Segment of Hybanthus Enneaspermus

Growth Regulators (mg/L)		Culture Response in Percentage	Callus Response
BA	NAA		
0.5	-	50	++
1.0	-	60	++
1.5	-	40	+
2.0	-	40	+
2.5	-	20	-
0.5	0.2	50	++
1.0	0.4	70	+++
1.5	0.6	40	+
2.0	0.8	40	+
2.5	1.0	20	-

0 – 20% - No Response (or) Very Less response, 21-40% + Poor Response, 41-70% ++ Moderate Response, 71-100% +++ Good Response

Table 5: Influence of Diverse Levle of 2,4-D and NAA on Callus Formation from nodal Segment of Hybanthus Enneaspermus

Growth regulators (mg/L)		Culture response in percentage	Callus response
2,4-D	NAA		
0.5	-	30	+
1.0	-	50	++
1.5	-	70	+++
2.0	-	60	++
2.5	-	30	+
0.5	0.2	30	+
1.0	0.4	60	++
1.5	0.6	80	+++
2.0	0.8	60	++
2.5	1.0	20	-

0 – 20% - No Response (or) Very Less response, 21-40% + Poor Response, 41-70% ++ Moderate Response, 71-100% +++ Good Response

Table 6: Influence of Diverse Level of BA and NAA on Callus Formation from Shoot tip Segment of Hybanthus Enneaspermus

Growth Regulators (mg/L)		Culture Response in Percentage	Callus Response
BA	NAA		
0.5	-	50	+
1.0	-	70	+++
1.5	-	60	++
2.0	-	40	+
2.5	-	20	-
0.5	0.2	60	++
1.0	0.4	80	+++
1.5	0.6	60	++
2.0	0.8	40	+
2.5	1.0	20	-

0 – 20% - No Response (or) Very Less response, 21-40% + Poor Response, 41-70% ++ Moderate Response, 71-100% +++ Good Response

Table 7: Influence of Diverse Level of 2,4-D and NAA on Callus Formation from Shoot Tip Segment of Hybanthus Enneaspermus

Growth regulators (mg/L)		Culture response in percentage	Callus response
2,4-D	NAA		
0.5	-	30	+
1.0	-	40	+
1.5	-	80	+++
2.0	-	60	++
2.5	-	20	-
0.5	0.2	30	+
1.0	0.4	50	++
1.5	0.6	90	+++
2.0	0.8	60	++
2.5	1.0	20	-

0 – 20% - No Response (or) Very Less response, 21-40% + Poor Response, 41-70% ++ Moderate Response, 71-100% +++ Good Response

CONCLUSIONS

The results of this study indicates that among the six pathogens tested *V. cholera* was most susceptible to the solvent extracts followed by *P. aeruginosa, B. subtilis, S. aureus, K. pneumonia,* and *E. coli.* Among the different solvents tested, Diethyl ether was more active and the rest of the extract showed moderate inhibitory effect to the test pathogens. Maximum callus induction was noted in MS medium fortified with BA (1.0mg/L) + NAA (0.4mg/L) from leaf explant of *Hybanthus ennaspermus. This antibacterial activity* may be used in the treatment of various diseases as well as suitable for use in the pharmaceutical and cosmetic industries. However, further research is required to find out the active compounds in crude extract for appropriate medication development.

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